HANSENULA POLYMORPHA YAPSIN DEFICIENT MUTANT STRAIN AND PROCESS FOR THE PREPARATION OF RECOMBINANT PROTEINS USING THE SAME

Technical Field

The present invention relates to a process for producing a recombinant protein secreted and expressed from methanol-utilizing *Hansenula polymorpha* yeast at a high yield by efficiently preventing decomposition of the recombinant protein.

More preferably, it relates to a process for efficiently producing a recombinant protein by destroying yapsin1 gene of *H. polymorpha* strain to prevent decomposition of the protein containing a basic or dibasic amino acid residue produced in *H. polymorpha*.

More particularly, it relates to a process for producing a recombinant protein in an intact configuration at a high yield in *H, polymorpha* by preventing decomposition of the recombinant protein having a basic or dibasic amino acid residue in the protein, such as human parathyroid hormone, human serum albumin and serum albumin fusion protein, which comprises cloning the *HpYPS1* gene encoding *Hansenula polymorpha* aspartic protease type yapsin1, preparing a *H. polymorpha* strain with the defective *HpYPS1* gene using the cloned gene and culturing a transformant transformed from the strain as a host.

Background Art

Recently, as demand for high-purity protein medicaments is suddenly increased due to increase of incurable diseases and improvement of public medical standard, relative importance of medicinal recombinant proteins in the health-related bioengineering field is highly raised.

Therefore, the frequency of use of yeast which is a monocellular eukaryotic microorganism as a host system for mass-production of a recombinant protein is gradually increased. Particularly, since yeast has the protein secretion route very similar to those of higher animal cells, it is habitually used as a microorganism host system for production of human-derived secretion proteins. Also, since most kinds of yeast normally secret a very small number of proteins out of the cell, advantageously, recombinant proteins secreted from yeast can be readily recovered and purified. In recent, mass-production of serum proteins, vaccines and other various important medical proteins using non-traditional yeasts including *Hansenula polymorpha* and *Pichia pastoris*, other than the traditional yeast *Saccharomyces cerevisiae* has been successively conducted (Gellissen G., Appl. Microbiol. Biotechnol. 54, 741 (2000)).

As a eukaryotic microorganism, yeast secretes proteins by the substantially same method with mammal cells and involves similar protein modification and cleavage procedures. A protein which has undergone the secretion route becomes to have its final 3-dimensional structure at the Endoplasmic reticulum. In case of glycoprotein, N- and O-

bonding sugar chains are attached thereto. Subsequently, the protein is transferred to the Golgi apparatus, in which it is further subjected to the protein modification procedures such as trimming of oligosaccharide or protein cleavage, and thereafter, is transferred to different organs, inserted into components of the cell membrane or secreted out of the cell.

As described above, since the protein secretion procedures in yeast involve various kinds of post-translational modification processes, the secretion and production of a foreign protein in yeast may cause many problems. Particularly, when a recombinant protein is secreted and produced in yeast, it is necessary to use an efficient expression and secretion system in order to increase productivity, but is also important to prevent decomposition of the produced and secreted foreign protein. If a recombinant yeast is cultured for a long period of time at a high concentration in a fermenter, proteases which are naturally secreted from the host cell or exist in the cell through cell lysis are released to medium and degrade the produced recombinant proteins, thereby causing reduction in overall productivity of the recombinant proteins. In order to solve this problem, for yeasts including *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris* and the like, which have been used as recombinant protein expression systems, various protease deficient strains have been developed. Primarily, strains, in which *PEP4, PRB1*, or *CPY* genes encoding degradative enzymes existing in yeast vacuole are destroyed (Alvarez et al., J. Biotechnol. 38, 81 (1994); Chen et al., Curr. Genet. 27, 201 (1995); Gleeson et al., Methods Mol. Biol. 103, 81 (1998); Kang et al. In *Hansenula polymorpha* (ed. Gellissen G.) p.124 (2001)), have been developed. In addition to the vacuole

degradative enzymes, *kex1*△ strain have been developed, in which *KEX1* gene encoding carboxypeptidase α existing in the Golgi apparatus is destroyed. By the *kex1*△ strain, C-end decomposition of hirudin in *Saccharomyces cerevisiae* (Hinnen et al., In Gene expression in recombinant microorganisms (ed. Smith A.), p121 (1995)), of human epidermal growth factor in *Hansenula polymorpha* (Heo et al., Protein expr. purif. 24, 117 (2001)) and of rodent or human endostatin in *Pichia pastoris* (Boehm et al., Yeast 15, 563-567 (1999)) can be significantly reduced.

Recently, yeast aspartic protease type yapsins having activity to recognize and cut basic amino acids existing as a single or a pair in *Saccharomyces cerevisiae* have been identified, which are novel proteases existing in the cell membrane (Egel-Mitani et al., Yeast 6, 127-137 (1990)). Yapsin1 (also previously known as yeast aspartic protease 3(YAP3)) was firstly known to the public among the yeast aspartic proteases, and yapsin2 (also previously known as MKC7) was known thereafter (Komano and Fuller, Proc. Natl. Acad. Sci. USA 7, 92,10752-10756 (1995)). By the *Saccharomyces cerevisiae* genome information which has been recently disclosed to the public, additional genes encoding at least 5 yapsin type protease presumed to have similar functions, such as yapsin3, yapsin6 and yapsin7, have been reported to exist so far (Olsen et al. Biochem. J. 339, 407-411 (1999)). Though the physiological functions of these yapsins are not clearly shown, as the number of study cases reporting that target recombinant proteins which are intended to secret and produce in *S. cerevisiae* are cleaved by the protease activity of yapsin is increased, yapsin deficient yeast strains attract public attention as an

useful strain for production of a recombinant protein, particularly a foreign peptide having a basic amino acid. Recombinant proteins which have been reported to have problems of being cleaved by yapsins in secretion and production in *S. cerevisiae*, till now, include human serum albumin (Kerry-williams et al., Yeast 14, 161-169 (1998)), human parathyroid hormone (Kang et al., Appl Microgiol Biotechnol., 50, 187-192 (1998)); Korean Patent Registration No. 0246932 (publicated on December 8, 1999)), insect diuretic hormone (Copley et al., Biochem J., 330, 1333-1340 (1998)), glucagon and glucagon-like peptide (Egel-Mitani et al., Enzyme Microb Technol. 26, 671-677 (2000): USA PAT. NO. 6,110,703) and human elafin precursor (Bourbonnais et al., Protein Exp. Purif. 20, 485 (2000)). Meanwhile, considering that *YPS1* deficient *S. cerevisiae* strain shows a considerable progress in decomposition of hPTH at the last stage of the cultivation using a fermenter, the present inventors have developed *S. cerevisiae* yapsin multiple deficient mutant strain (*yps1*△/*yps2*△/*yps3*△), in which the *YPS2* and *YPS3* genes coding for yapsin2 and yapsin3 are removed. As a result, we have obtained an excellent result of preventing 90% or more of degradation of human parathyroid hormone observed in a high-concentration cultivation (Korean Patent Application No. 2000-51267 and International Application No. PCT/KR01/01447).

H. polymorpha , one of methanol-utilizing yeasts, is in the spotlight as a very useful yeast host for mass production of recombinant proteins since it has advantages in that strong and controllable promoters are developed, alike *Pichia pastoris,* and a foreign gene can be multiply introduced into the host chromosome (Faber et al., Yeast 11,

1331(1995)). Up to date, various kinds of foreign proteins have been expressed and the expression levels often reached over 1 g/L in case of high-concentration cultivation using a fermenter. Particularly, it has been reported that when recombinant phytase is secreted and produced, the expression level is about 13.5 g/L (Mayer et al., Biotechnol. Bioeng. 63, 373-381 (1999)). Therefore, the *H. polymorpha* expression system becomes distinguished as one of the most potential systems among several presently available eukaryotic cell expression systems. Especially, since some of the recombinant proteins which have been produced in the initial stage in *H. polymorpha* have already passed clinical trials and are on the market (ex., hepatitis B vaccine) or in the product development phase(ex., hirudin), *H. polymorpha* is considered as a suitable expression system for production of a recombinant protein to be developed as an medicament (Gellissen G., Appl Microbiol Biotechnol. 54 741-750 (2000)). Also, as recently getting into the post-genome era, there is an increased need for a high-efficiency expression system for functional analysis of novel genes, and thus it is expected that an expression system using *H. polymorpha* would bear a great part in functional and structural analysis of novel proteins as well as mass production of useful proteins derived from higher eukaryotic cells.

Disclosure of Invention

Therefore, the present invention has been made to develop a high-efficiency *H.*

polymorpha expression system by solving the problems related to undesired cleavage and decomposition of a recombinant protein by an yapsin type protease, and it is an object of the present invention to provide a technology for secreting and producing a recombinant protein in an intact configuration at a high efficiency by preventing cleavage and decomposition of the recombinant protein expressed in *H. polymorpha*, in which the *HpYPS1* gene encoding *H. polymorpha* yapsin1 is cloned, the cloned gene is used to prepare *HpYPS1* gene deficient mutant strain and the prepared mutant strain is used as a recombinant protein expression host.

In accordance with an aspect, the present invention provides a gene sequence encoding yapsin1(*HpYPS1*) of *H. polymorpha* strain by probing yapsin protease genes in *Hansenula polymorpha* strain, as identified in *S. cerevisiae*.

Also, in further aspect, the present invention provides a yapsin1 polypeptide having *Hansenula polymorpha* strain-derived aspartic protease activity.

Also, in another aspect, the present invention provides a secretion signal gene sequence and a peptide sequence of *HpYPS1* polypeptide for secretion of a foreign protein recombinantly produced in *Hansenula polymorpha* strain.

According to yet another aspect, in order to develop a *Hansenula polymorpha* strain capable of secreting and producing a recombinant protein in an original configuration at a high efficiency, the present invention provides an expression system for secretion and production of a recombinant protein at a high efficiency in *Hansenula polymorpha* by cloning the above-described *Hansenula polymorpha* yapsin protease gene

and deleting the cloned gene to minimize the decomposition of the recombinant protein in *Hansenula polymorpha*.

In order to accomplish the above objects, the present inventors have developed a *Hansenula polymorpha* mutant strain (*hpyps1*△), in which the *HpYPS1* gene is deleted, by cloning the *HpYPS1* gene encoding *Hansenula polymorpha* yapsin1 and subjected the resulting *HpYPS1* gene to a functional analysis. Consequently, we have formed a method for increasing productivity of a recombinant protein by using the *Hansenula polymorpha* mutant strain as a host strain to express the protein containing a basic or dibasic amino acid residue in the protein, including human parathyroid hormone, human serum albumin and human serum albumin fusion protein, thereby significantly reducing the decomposition of the recombinant protein expressed and secreted in *H. polymorpha*.

Brief Description of Drawings

Further objects and advantages of the invention can be more fully understood from the following detailed description taken in conjunction with the accompanying drawings in which:

Fig. 1 shows the nucleotide sequence of gene *HpYPS1* encoding *H.polymorpha* yapsin1 and the expected amino acid sequence (①: a signal sequence, ②: the expected GPI anchor, ③: a hydrophobic amino acid-golgi membrane fixing domain);

Fig. 2 shows the comparison of the amino acid sequence between *H.polymorpha*

yapsin1 and *S. cerevisiae* yapsin type proteases;

Fig. 3 is the result of the functional complementation experiment showing that temperature sensitivity of *Saccharomyces cerevisiae* yapsin multiple deficient mutant strain is recovered as the *Hansenula polymorpha* yapsin1 gene *HpYPS1* is expressed;

Fig. 4 is a schematic view showing the procedures for preparing a *Hansenula polymorpha* yapsin1 gene -disrupted mutant strain *hpyps1*△;

Fig. 5 shows the results of comparison of protease activity in the culture supernatants of the *H. polymorpha* wild type and the yapsin1-deficient *hpyps1*△mutant strain, in which human parathyroid hormone is used as a substrate;

Fig. 6 shows a schematic view of the procedures to construct the human parathyroid hormone expression vector pMOXhPTH for *H. polymorpha*;

Fig. 7A shows the result of Southern blot to examine the expression vector insertion sites and the number of copies by isolating chromosomes of the *Hansenula polymorpha* wild type and the mutant strain *hpyps1*△ transformed with the vector pMOXhPTH;

Fig. 7B shows the results of SDS-polyacrylamide electrophoresis of yeast culture supernatants, followed by staining, to compare the expression and decomposition aspects of recombinant human parathyroid hormone in the transformants of *H.polymorpha* wild type and *hpyps1*△ mutant strain;

Fig. 8A shows the results of comparison of the expression and decomposition aspects of recombinant human serum albumin when the *H. polymorpha* wild type strain

and the *HpYPS1* gene-deleted *hpyps1*△ mutant strain are used as a host, in which yeast culture supernatants are analyzed by SDS-polyacrylamide electrophoresis, followed by staining;

Fig. 8B shows the results of comparison of the expression and decomposition aspects of recombinant human serum albumin when the *H. polymorpha* wild type strain and the *HpYPS1* gene-deleted *hpyps1*△mutant strain are used as a host, in which yeast culture supernatants are analyzed by Western blotting;

Fig. 9 shows a schematic view of the procedures for constructing the albumin-TIMP2 fusion protein expression vector pYHSA13-T2 for *Hansenula polymorpha*; and

Fig. 10 shows the results of the comparison of the expression and decgradation aspects of recombinant albumin-TIMP2 fusion protein when the *H.polymorpha* wild type strain and the *HpYPS1* gene-deleted *hpyps1*△ mutant strain are used as a host, in which yeast culture supernatants are analyzed by Western blotting. The Lane 1 represents the wild type transformant transformed with pYHSA12(+), the Lanes 2 to 5 represent the wild type transformant transformed with pYHSA13-TIMP2, the Lanes 6 to 9 represent the *hpyps1*△mutant strain transformed with pYHSA13-TIMP2 and the Lane 10 represent isolated and purified albumin (200 ng).

Best Mode for Carrying Out the Invention

H. polymorpha is a class of yeast which can use methanol as a carbon source

and energy source. According to the present invention, there is provided a *H. polymorpha* mutant strain with yapsin enzyme destroyed, based on the fact that when *H. polymorpha* is used in a method for producing a foreign protein, the yield of the foreign protein is reduced since the produced foreign protein is decomposed by yapsin enzyme which is an aspartic protease existing in the yeast.

As described herein, the yapsin enzyme family, which is a sub-family of aspartic proteases, has an ability to specifically cleave a basic or dibasic amino acid residue of a protein. This is a difference from other aspartic proteases cleaving hydrophobic residues.

The present inventors have obtained a segment showing similarity with the *S. cerevisiae* yapsin1 encoding gene (*YPS1*) from the PCR-amplified chromosomes derived from *H. polymorpha* and conducted researches and studies on functions of the segment.

As a result, the segment was identified as *HpYPS1* gene of *H. polymorpha*. Further, we have prepared a *Hansenula polymorpha* mutant strain having the *HpYPS1* gene destroyed and confirmed that a foreign protein can be produced from the strain at a high yield. Thus, the present invention has been completed.

In one aspect, the present invention provides a nucleic acid molecule comprising the sequence shown in Fig. 1.

More particularly, the present invention provides a nucleic acid molecule comprising the sequence encoding *Hansenula polymorpha* yapsin1 shown in Fig. 1.

In another aspect, the present invention provides a polypeptide comprising the amino acid sequence shown in Fig. 1.

More particularly, the present invention provides a polypeptide comprising the amino acid sequence of *Hansenula polymorpha* yapsin1(HpYPS1) shown in Fig. 1.

In another aspect, the present invention provides a secretion signal gene sequence and a peptide sequence of HpYPS1 polypeptide for secretion of a foreign protein recombinantly produced in *Hansenula polymorpha* strain, shown as ① in Fig. 1.

In another aspect, the present invention provides a *H. polymorpha* mutant strain having reduced yapsin activity by mutation of the *HpYPS1* gene encoding *H. polymorpha* yapsin1.

In another aspect, the present invention provides a recombinant *H.polymorpha* strain expressing a foreign protein by introducing a gene encoding the foreign protein into the *H. polymorpha* mutant strain.

In another aspect, the present invention provides a process for preparing a foreign protein comprising culturing a recombinant *H. polymorpha* strain under conditions to express the foreign protein and isolating the foreign protein from the culture.

More particularly, the present invention provides a process for secreting and producing a recombinant protein in *Hansenula polymorpha* using a yeast strain lacking the *HpYPS1* gene for a yapsin type protease as a host. The present invention comprises the steps of: cloning gene the *HpYPS1* encoding *H. polymorpha* protease yapsin1; functional analyzing the resulting *H. polymorpha HpYPS1* gene by temperature sensitivity complementation experiment of a *Saccharomyces cerevisiae* yapsin multiple deficient mutant strain; preparing a *hpyps1Δ* mutant strain with the *HpYPS1* gene

destroyed in *Hansenula polymorpha*, followed by analysis of yapsin activity; and preparing a recombinant *Hansenula polymorpha* strain *hpyps1 Δ*-pMOXhPTH to express and secrete human parathyroid hormone, a recombinant *Hansenula polymorpha* strain *hpyps1 Δ*-pYHSA12 to express and secrete human serum albumin, or recombinant a *Hansenula polymorpha* strain *hpyps1 Δ*-pYHSA13-TIMP2 to express and secrete a TIMP2 protein fused with human serum albumin recombinant protein using the *H .polymorpha* mutant strain *hpyps1 Δ* as a host, followed by analysis of decomposition level of the recombinant protein. Therefore, the present invention relates to a method for efficiently producing a recombinant protein by preventing degradation of the recombinant protein expressed from methanol utilizing yeast *H. polymorpha*, which comprises cloning the *HpYPS1* gene encoding *H. polymorpha* aspartic protease type yapsin, preparing a *H. polymorpha* strain with the *HpYPS1* gene deficient using the cloned gene, and culturing a transformant transformed from the strain as a host to minimize degradation of the recombinant protein, thereby producing the recombinant protein in an intact configuration at a high yield.

The sequence of the *H. polymorpha YPS1* gene (*HpYPS1*) (SEQ ID NO: 1) cloned according to the present invention was deposited in GenBank under Accession No. AF493990. Also, the *HpYPS1* gene encoding *H. polymorpha* protease yapsin1 was deposited in an international depository authority (Korean Collection for Type Cultures) on the date of June 18, 2002 and assigned Accession No. KCTC 10285BP. Further, the *hpyps1 Δ* mutant strain having the *HpYPS1* gene destroyed in *H. polymorpha* was

deposited in an international depository authority (Korean Collection for Type Cultures) on the date of June 18, 2002 and assigned Accession No. KCTC 10281BP. In an embodiment, the recombinant *H, polymorpha* strain *hpyps1* Δ-pMOXhPTH expressing and secreting human parathyroid hormone was deposited in an international depository authority (Korean Collection for Type Cultures) on the date of June 18, 2002 and assigned Accession No. KCTC 10282BP. In another embodiment, the recombinant *H. polymorpha* strain *hpyps1* Δ-pYHSA12 expressing and secreting human serum albumin was also deposited in an international depository authority (Korean Collection for Type Cultures) on the date of June 18, 2002 and assigned Accession No. KCTC 10283BP. In addition, the recombinant *H. polymorpha* strain hpyps1 Δ-pYHSA13-T2 expressing and secreting albumin-TIMP2 fusion protein was deposited in an international depository authority on the date of June 23, 2003 and assigned Accession No. KCTC 10485BP.

Now, the present invention will be explained in detail by the following examples. However, it should be understood that the following examples are only for illustrative purposes and are not intended to limit the scope of the invention.

<Example 1>

Cloning of *Hansenula polymorpha* yapsin1 gene *HpYPS1* and analysis of the sequence

In order to find a gene encoding yapsin1 in *H. polymorpha*, a pair of synthetic oligonucleotides (5`-GAAGTGCAGCAGCAGCTCCTGAACC-3'; SEQ ID NO: 3, 5`-

GGCTGATGACGGCTCGGTCACGATGG-3'; SEQ ID NO: 4) were prepared, on the basis of information on *H. polymorpha* random sequenced tags described in Blandin et al., (FEBS Lett. 487, 76, (2000). By PCR (Polymerase Chain Reaction) using the prepared oligonucleotides as primers, a 0.88 kb DNA segment was amplified from *H. polymorpha* DL-1L derived chromosome. Then, Southern blotting was conducted using the amplified DNA segment as a probe. Based on the result of the Southern blot, 3.5 kb HindIII DNA segments was extracted from *H. polymorpha* chromosomal DNA to prepare a genome library, which was transformed into *E. coli.* A DNA segment reactive with the DNA probe was isolated by colony PCR and subjected to DNA sequencing to identify a DNA segment comprising ORF (Open Reading Frame) with a size of1728 bp showing a high similarity with the *Saccharomyces S. cerevisiae YPS1* gene (Fig. 1). The *H. polymorpha YPS1* gene (*HpYPS1*) product has a signal sequence of 1-17 amino acids at its N-end and a region of 556-575 amino acids, presumed as a domain which can be anchored on the membrane of Golgi apparatus, as reported on *S. cerevisiae* yapsin1.

Also, it has structural features, by which a glycosylphosphatidylinositol anchor can be attached thereto, as in *S.cerevisiae* yapsins (Fig. 1). *H. polymorpha* yapsin1 (SEQ ID NO: 2) shows a homology of 36% and a high similarity of 52% with *S. cerevisiae* yapsin1 and a homology of 30% or more with other yapsin proteases (Fig. 2, Table 1).

Table 1

Homology and similarity of *HpYPS1* and *ScYPS* genes

	ScYPS1	*ScYPS2*	*ScYPS3*	*ScYPS6*	*ScYPS7*
HpYPS1	36%(52%)	31%(49%)	30%(44%)	26%(44%)	29%(34%)

* Parenthesized number is similarity

<Example 2>

Functional analysis of the *Hansenula polymorpha HpYPS1* gene

In case of the traditional yeast *Saccharomyces cerevisiae*, it has been reported that *YPS1/YPS2* double deficient mutant strain derived from *S. cerevisiae* W303 strain as a parental strain shows a such high temperature sensitivity that it cannot grow at 37℃ (Komano and Fuller, Proc. Natl. Acad. Sci. USA 7,92,10752-10756 (1995)). Also, SLH18(*yps1*△/*yps2*△/*yps3*△) strain (Korean Patent Registration No. 10-0386836), which is a yapsin multiple deficient mutant strain having three genes of *YPS1/YPS2/YPS3* destroyed, prepared using *Saccharomyces cerevisiae* L3262 as a parental strain by the present inventors, was found to show temperature sensitivity at a high temperature. As a method for functional analysis of the *Hansenula polymorpha* yapsin1 gene *HpYPS1*, a functional complementation experiment was conducted by transforming the *HpYPS1* gene into the above-described *Saccharomyces cerevisiae* multiple yapsin mutant strain according to the lithium chloride-DMSO method (Hill et al., Nucleic Acid Res., 19, 5791 (1991)) to examine whether the expression of *Hansenula polymorpha* yapsin1 gene could restore temperature sensitivities of the *Saccharomyces cerevisiae* multiple yapsin mutant

strains. In order to express the *HpYPS1* gene in *Saccharomyces cerevisiae*, 3.2 kb DNA segment including the *HpYPS1* gene was inserted into the *Saccharomyces cerevisiae* multicopy vector YEp352 (Hill et al., Yeast 2, 163, (1986)) to generate YEp-*HpYPS1*.

As shown in Fig. 3, the *Saccharomyces cerevisiae* yapsin multiple deficient mutant strains transformed with the YEp352-*HpYPS1* vector containing the *HpYPS1* gene showed the same growth with the wild type strain at 37℃, while the yapsin multiple deficient mutant strain transformed with only YEp352 vector, used as control, did not grow at 37℃. From these results, it was proved that the temperature sensitivity due to the multiple deletion of *Saccharomyces cerevisiae* yapsin genes can be overcome by the expression of the *Hansenula polymorpha* yapsin 1 gene. Thus, there was provided a basis supporting that the *H. polymorpha* gene *HpYPS1* cloned according to the present invention is a functional homologue of yapsin gene of *S. cerevisiae*.

<Example 3>

Preparation of the *HpYPS1* gene-deficient strain and analysis of yapsin activity

Using the *HpYPS1* gene prepared above, a *H. polymorpha* yapsin deficient mutant strain *hpyps1*△ was synthesized and assayed for change in its protein decomposition activity by yapsin. In order to prepare a mutant strain having the deletion of the *HpYPS1* gene encoding *Hansenula polymorpha* yapsin 1, a fusion PCR (Oldenhurg et al., Nucleic Acid Res. 25, 451, (1997)) was conducted on *H. polymorpha* chromosomal DNA using primers described in Table 2. The resulting DNA segment was transformed

into *Hansenula polymorpha* DL1-LdU (leu2△ura3::lacz; Kang et al., In *Hansenula polymorpha*:Biology and Application (Ed. G. Gellissen), pp 124 (2002)) strain to induce *in vivo* homologous recombination, thereby attempting deletion of the gene. More specifically, a primary PCR was firstly conducted to obtain the N-ends, C-ends of *HpYPS1* gene and URA3 gene. Then, a secondary fusion PCR was conducted to fuse the N-end and C-end of *HpYPS1* gene with the N-end and C-end of URA3 gene, respectively. The two DNA segments thus obtained were introduced to yeast cells.

Using *URA3* as a selection marker, viable transformants were selected in a minimal medium lacking uracil. Construction of the *hpyps1*△ strain (*leu2*△*ura3::lacz yps1:: URA3*), which is a *H. polymorpha* mutant strain having the deleted *HpYPS1* gene, was confirmed by PCR, which generated the DNA segments with different sizes between the wild type strain and the mutant strain. (Fig. 4).

For the comparison of the yapsin activity of *H. polymorpha hpyps1*△ mutant strain prepared above with that of the wild type strain, each of the two strains was cultured for 10 hours in YPD medium (1% yeast extract, 2% peptone, 2% glucose) using human parathyroid hormone (hPTH) as a substrate. The resulting yeast culture supernatant was analyzed for its protein degradation activity. 20 ㎕ of the yeast culture supernatant which had been diluted 1/4 was mixed with hPTH (about 1.6 ㎍) acting as a substrate. The reaction was incubated for 2 hours, 4 hours and 6 hours at 37℃. Each product was loaded on SDS-polyacrylamide gel to analyze the degradation level of hPTH by the yapsin activity.

As shown in Fig. 5, in the culture fluid of the wild type, it was already hard to observe hPTH remaining in the culture supernatant after 2 hour cultivation, while in the culture supernatant of *hpyps1*△ mutant strain, a substantial amount of hPTH was observed, though the amount was somewhat reduced as the reaction time became longer.

This suggests that the culture supernatant of *hpyps1*△, the *Hansenula polymorpha HpYPS1* deficient mutant strain, had a significantly reduced hPTH degradation activity, as compared to the culture supernatant of the wild type strain. Such result shows the same result with the previous study performed on *S. cerevisiae YPS1* deficient mutant strain (Kang et al., Appl Microbiol Biotechnol. 50, 187, (1998)) and thus, supports the fact that *HpYPS1* is a gene encoding yapsin protease of *H. polymorpha* and the *HpYPS1* deficient mutant strain developed according to the present invention has the yapsin activity reduced.

Table 2

Primers used in fusion PCR for destruction of *HpYPS1* gene

Primer	Sequence	SEQ ID NO:
YPS(NF)	5'-GGACACGCAAGAGGTGTCTG- 3'	5
YPS(NR+rp)	5'-AGCTCGCTACCCGGGGATCCGCAACTTTCATTGTGTCAAC- 3'	6
YPS(CF+rp)	5'-GCACATCCCCCTTTCGCCAGCCTCTTCGGTGCGGTTGACC- 3'	7
YPS(CR)	5'-GCTCGGCTCCAGGATTCAGG- 3'	8
URA3 N-S	5'-GGATCCCCGGGTACCGAGCT- 3'	9

URA3 N-A	5'-CACCGGTAGCTAATGATCCC- 3'	10
URA3 C-S	5'-CGAACATCCAAGTGGGCCGA- 3'	11
URA3 C-A	5'-CTGGCGAAAGGGGGATGTGC- 3'	12

<Example 4>

Construction of the recombinant *H. polymorpha* strain expressing hPTH and analysis of the hPTH expression

In order to practically express and secret human parathyroid hormone (hPTH) in *H. polymorpha*, pMOXhPTH, a human parathyroid hormone expression vector for *H. polymorpha* was prepared according to a method shown in Fig. 6. Thus, the 0.53 kb EcoR I /Sal I segment containing the hPTH cDNA fused with a MFα signal sequence was prepared from pG10-hPTH , a hPTH expression vector for *S. cerevisiae* (Chung et al., Biotechnol Bioeng. 57, 245, (1998)) and the 7.8 kb Xba I /EcoR I segment having the albumin cDNA removed was prepared from pYHSA12, a human serum albumin expression vector for *H. polymorpha* (Kang et al., Biotechnol Bioeng. 76, 175, (2001)).

The two DNA segments were joined to generate pMOXhPTH. The resulting vector pMOXhPTH was introduced into the *H. polymorpha hpyps1*△ mutant strain and the wild type strain, and viable transformants were selected in minimal medium lacking leucine. At every 24 hours after inoculation, Leu^+ transformants were transferred to liquid minimal selective medium lacking leucine 5 times to stabilize the Leu^+ transformants (Sohn et al., Appl Microbiol Biotechnol. 51, 800, (1999)). The culture broth of the Leu^+ transformants obtained as described above was plated onto minimal

media containing G418 at various concentrations and cultured at 37℃. DNA was isolated from each of the resulting colonies and subjected to Southern blot analysis using the 1.5 kb *H. polymorpha LEU2* gene as a hybridization prove according to the method described by Sambrook et al. (Molecular cloning Cold Spring Harbor Laboratory Press, 1989) to confirm insertion into the chromosome of the expression vector (Fig. 7A). The *H. polymorpha LEU2* gene used as a probe was prepared by labeling with digoxigenin using the non-radioactive DNA labeling and detection kit. As having been expected, in the transformants selected on the medium containing a high G418 concentration, multiple integration of the vector pMOXhPTH was observed. Upon comparison of intensity between the *LEU2* gene signal on the chromosome and the *LEU2* gene signal on the inserted vector, it was presumed that about 5 to 6 copies at most had been inserted.

Some tranformants (a, b, c, d, e and f) of the recombinant yeast strains which had been confirmed to have the hPTH expression vector integrated into the chromosome were inoculated into YPM medium (1% yeast extract, 2% peptone, 2% methanol) and cultured at 37℃. Yeast culture supernatants obtained at 12 hours and 24 hours after initiation of the cultivation were treated with TCA (trichloroacetic acid). The proteins secreted out of the cells were concentrated to 1/20 of the initial volume and electrophoresed on 15% SDS-polyacrylamide gel, followed by staining with Coomassie Brilliant Blue R-250 (Fig. 7B).

When the hPTH secreted in *polymorpha* wild type was compared with that secreted in the *hpyps1*△ mutant strain, only a trace of decomposition product (d1) was

observed in the culture supernatant obtained from the wild type and hPTH of a whole size (i) was hardly observed, since most of hPTH was decomposed. On the other hand, secretion of hPTH of a whole size was clearly observed in the culture supernatant obtained from the *hpyps1*△ mutant strain at 12 hours after cultivation. From these results, it was shown that the *hpyps1*△ mutant strain having the yapsin1 gene destroyed is a more useful strain as a host for secretory expression of recombinant hPTH, as compared to the wild type, since the decomposition of the recombinant parathyroid hormone is considerably inhibited by the reduction of yapsin activity, although hPTH of a whole size was reduced in the *hpyps1*△ mutant strain while the band (d2), it is presumed, of hPTH having the C-end decomposed was observed, as the cultivation time became longer.

<Example 5>

Analysis of expression and secretion of recombinant human serum albumin in *H. polymorpha HpYPS1* gene deficient mutant strain

The human serum albumin (HSA) expressed in *S. cerevisiae* is secreted as an intact form of 67 kDa, however some recombinant HSA in a decomposed form of 45 kDa also have been observed. It was reported that degradation of HSA secreted to yeast cell culture supernatant, particularly production of a decomposition product with a size of 45 kDa was reduced when a *S. cerevisiae* strain with the yapsin1 gene destroyed was used as a host (Kerry Williams et al., Yeast 14, 161, (1998)). In order to analyze the expression

and degradation aspects of recombinant HSA in the *H. polymorpha HpYPS1* gene deficient mutant strain developed according to the present invention, the *hpyps1*△ mutant strain was transformed with an expression vector pYHSA12 (Kang et al., Biotech Bioeng. 76, 175, (2001)), in which the *MOX* promoter and the HSA cDNA were joined to a vector for copy-number controlled gene integration using the *LEU2* gene and G418 resistance gene as selective markers. Recombinant *H. polymorpha* wild type strain and *hpyps1*△ mutant strain were cultured in YPGM medium (1% yeast extract, 2% peptone, 1% glycerol, 2% methanol). Each of the culture supernatants thus-obtained was loaded on SDS-polyacrylamide gel, followed by staining with silver nitrate, or transferred to a nitrocellulose membrane, followed by Western blot by HSA antibody to examine expression and degradation aspects of HSA. As shown in Fig. 8, it was observed that more HSA was secreted in the *hpyps1*△ mutant strain than the wild type strain, particularly after cultivation for 24 hours. Though the overall degradation of albumin was not significantly inhibited, it was shown that degradation product with a size of 45 kDa was apparently reduced in the *hpyps1*△ mutant strain, as compared to the wild type, as observed particularly in *S. cerevisiae*. Considering cultivation in a high concentration fermenter, production of 45 kDa degraded HSA product presents more serious problems, it is expected that an albumin production system using the *hpyps1*△ mutant strain as a host can increase production of albumin due to a remarkable reduction in degradation of albumin, as compared to an production system using the wild type strain as a host.

<Example 6>

Analysis of expression and secretion of recombinant albumin fusion TIMP2 (HSA-TIMP2) in *H. polymorpha HpYPS1* gene deficient mutant strain

As a part of a method to increase blood persistence of a protein therapeutic agent, researches to develop a technology to increase *in vivo* half-life of a medical protein by expressing a recombinant protein in a form fused to albumin, a blood protein which has a long stability, are in the spotlight (Smith et al., Bioconjugate Chem. 12, 750-756, (2001); Sheffield et al. Blood Coagul Fibrinol. 12, 433-443, (2001)). The present inventors also have developed a recombinant TIMP-2 having *in vivo* stability significantly increased by expressing TIMP-2, which attracts public attention as a next generation anti-angiogenesis agent and anti-tumor agent, as a recombinant protein fused to albumin in *S. cerevisiae* (Korea Patent Registration No. 10-2001-0027823, International Application No. PCT/KR03/00015). In order to analyze the expression and e decomposition aspect of the recombinant HSA-TIMP2 in a form fused to albumin in the *H. polymorpha HpYPS1* gene deficient mutant strain (*hpyps1*△) developed according to the present invention, YHSA13-T2, a HSA-TIMP2 expression vector for *H. polymorpha* was prepared according to the method shown in Fig. 9. That is, the DNA fragments encoding HSA and TIMP2 were prepared using the PCR primers described in Table 3. Fusion PCR was conducted using the prepared genes in a ratio of 1:1 to prepare the 2.4 kb *HSA-TIMP2* DNA segment comprising HSA (1.8 kb) and TIMP2 (0.586 kb), which are connected to each other. The resulting segment was cloned into pGEM T vector

(Promega, USA) to prepare pTHSA-TIMP2. The sequence of the HSA-TIMP2 DNA segment was confirmed by sequencing analysis. Then, pTHSA-TIMP2 was cut with *Xba*I and *Spe*I. The resulting *Xba*I/*Spe*I HSA-TIMP2 gene segment of 1.2 kb was joined to the XbaI-digested pYHSA12(+), a *H. polymorpha* multiple tandem introduction vector having albumin gene inserted (Kang et al., *Biotechnol. Bioeng.* 76, 175-185, (2001)), to prepare pYHSA13-T2 (Fig. 9).

Table 3

PCR primers used in preparation of HSA-TIMP2 fusion gene

primer	Sequence	Note
HSA EcoR F	5' gaattcatgaagtgggtaaccttt 3' (SEQ ID NO: 13)	HSA forward direction
Hs-R2	5' taagcctaaggcagcttgac 3' (SEQ ID NO: 14)	HSA reverse direction
H-T2-F	5'caagctgccttaggcttatgcagctgctccccggtg 3' (SEQ ID NO: 15)	Timp2 forward direction, used with 18 bp at HSA C-end in fusion PCR
R-T2-Sp	5' actagtgatttatgggtcctcgatg 3' (SEQ ID NO: 16)	Timp2 reverse direction

The albumin fusion protein expression vector prepared above was introduced into *H. polymorpha* DL1-L (leu2) and the *hpyps1*Δ *(leu2 hpyps1::URA3)* mutant strain to prepare Leu^+ transformants. The collected transformants were passage-cultured five times on selective medium so that the expression vectors could be multiply integrated to the host chromosomal DNA. Then, the transformants were plated at a density of $1x\ 10^5$ to 10^6 per plate in media containing antibiotic G418 at various concentrations to select

transformants having resistance to G418. In order to examine whether the transformants, which had been confirmed to have the expression vector pYHSA13-T2 integrated into the host chromosome by Western blotting, secrets and expresses the fusion protein with an expected size, the yeast culture supernatants obtained by culturing the transformants for 48 hours in YPM was analyzed by Western blotting using antibody to albumin. As shown in Fig. 10, it was observed that the fusion protein HSA-TIMP2 (88 kDa) having an increased size as compared to HSA (66.5 kDa) was secreted as expected. Interestingly, in case of the yapsin deficient mutant strain *hpyps1*Δ, only 88 kDa HSA-TIMP2 with a whole size was observed without any decomposition product. However, in the wild type DL1 strain, a band being presumed as a decomposition product was observed just under the band of HSA-TIMP2 with a size of 88 kDa. Therefore, due to the reduction of decomposition products, the expressed amount of HSA-Timp2 fusion protein was about two times higher in the *hpyps1*Δ mutant strain than the wild type strain. This suggests that protein degradation by yapsin1 is inhibited in secretion of recombinant proteins expressed as an albumin-fusion form as well as secretion of the above-described recombinant albumin in the *hpyps1*Δ mutant strain

Industrial Applicability

The present invention can be usefully used in bioengineering industry to produce a recombinant protein using *H. polymorpha* since cleavage of the recombinant protein by

yapsin activity can be remarkably reduced by using *H. polymorpha* strain (*hpyps1*△) with the protease yapsin1 gene being deficient as a recombinant protein-producing host, thereby secreting and producing the recombinant protein in an intact configuration at a high yield.

5

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#52, Oun-dong, Yusong-ku, Taejon 305-806,
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Date of deposit	Accession Number
18/06/2002	KCTC 10281BP

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